Carotid intima-media thickness and oxidative stress markers for assessment of atherosclerosis in children with β thalassemia major

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Abstract

The present study evaluates carotid intima-media thickness (CIMT) in children with β thalassemia major to assess atherosclerosis and its relation to the underlying proposed causative mechanisms *via* lipid peroxidation product malondialdehyde (MDA), oxidized low-density lipoproteins (LDL), total antioxidant level, and lipid profile. A cross sectional study was conducted on 62 children (31 cases and 31 controls). CIMT by high resolution ultrasound and biochemical parameters *i.e.*, total cholesterol, triglycerides, high-density lipoproteins, LDL, Oxidized LDL, lipoprotein (a), lipid peroxidation product MDA and total antioxidant were measured in enrolled subjects and compared. In our study, CIMT was significantly increased in β thalassemia major patients' as compared to healthy controls. Mean CIMT in cases was 0.69±0.11 mm and in controls 0.51±0.07 mm. Mean oxidized LDL (EU/mL) in cases 39.3±34.4 (range 14.4 to 160) was significantly raised (P=0.02, t test) as compared to controls 23.9±13.4 (range 12 to 70). In our study we found MDA levels (nmol/mL) to be increased in β thalassemia patients as compared to controls. Mean MDA was 10.0±3.27 (4.41 to 17.48) in cases while in controls was 6.87±4.55 (1.5 to 17.9). Our study results show CIMT as an early marker of atherogenesis in β thalassemia major. Oxidative stress markers are also increased in β thalassemia major patients and lipoprotein (a) shows a positive correlation with CIMT. The present study points towards various atherogenetic mechanisms in β thalassemia major.

Introduction

Thalassemia syndromes, is an autosomal recessive single gene disorder of hemoglobin chain synthesis. Every year approximately 100,000 children with thalassemia major are born world over, of which 10,000 are born in India.[1]

In β thalassemia, inadequate β globin chain production leads to decreased levels of normal adult hemoglobin (Hb A) and excessive production of α chains. This leads to precipitation of α chains, which leads to hemolysis and ineffective erythropoiesis. Ineffective erythropoiesis constitutes the main patho-physiology behind all signs, symptoms and complications of β-thalassemia.[2] Clinically β thalassemia major patients have symptoms of severe progressive hemolytic anemia typically presenting around 2-6 months of age. Treatment of patients with β thalassemia major includes regular lifelong blood transfusions and iron chelation. Iron overload occurs universally in all β thalassemia major patients over the disease course and results in varied complications. Iron overload in these patients result from ongoing chronic hemolysis, repeated blood transfusions, ineffective erythropoiesis and increased iron absorption from gastrointestinal tract.[3,4] Of the various complications, cardiovascular complications are the leading cause of morbidity and mortality in patients with β thalassemia major.[5] Currently, cardiac complications contribute to cause 71% of deaths in patients with thalassemia major.[6]

Atherosclerosis is an important forerunner of cardiovascular complications. Atherosclerosis in β thalassemia major patients may be the result of iron-overloaded state causing oxidative modification of lipids, altered lipid profile and vascular dysfunction. Studies have also shown relation between iron load and risk of atherosclerosis.[7] Iron overload is associated with generation of oxygen-free radicals and peroxidative tissue injury.[7] Oxidative modification of low-density lipoprotein (LDL) plays a central role in the sequence of events leading to atherogenesis-related vascular alterations. Malondialdehyde (MDA), a by-product of lipid peroxidation is found to be increased in patients with β thalassemia major.[8] So, plasma MDA levels in β thalassemia patients represent a sensitive index of the oxidative status of LDL *in vivo*.[8] Many studies have shown alterations in blood lipid profile in thalassemia major and intermedia patients.[9,10] Thalassemia patients also have altered endothelial relaxation, intimal thickening, abnormal vascular stiffening, and degeneration of elastic arteries.[3] Continuous blood transfusions along with iron overload leading to endothelial dysfunction *via* peroxidative tissue injury has also found to be an important precursor of atherosclerosis in patients with β thalassemia.[11]

Carotid intima-media thickness (CIMT) is a non-invasive method to detect early subclinical atherosclerosis and it correlates well with overall vascular injury and extend and severity of coronary artery disease.[7,12] There is upcoming role of CIMT for detection of early atherosclerosis in children with β thalassemia major.[13]

The present study evaluates CIMT in children with β thalassemia major to assess atherosclerosis and its relation to the underlying proposed causative mechanisms *via* lipid peroxidation product malondialdehyde (MDA), oxidized LDL, total antioxidant level, and lipid profile. There is no such study, to the best our knowledge, correlating CIMT with lipid peroxidation products and antioxidant levels.

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Materials and Methods

Study population

A cross-sectional study was conducted in Department of Pediatrics in collaboration with Department of Radiodiagnosis and Department of Biochemistry, Govt. Medical College and Hospital, Chandigarh. Sample size was calculated with α error of 0.05 and power of 80%, β error of 0.2, effect size (CIMT) from previous studies being 0.05 and standard deviation of 0.07. After approval from Institutional Ethical Committee and written informed consent from participants, 62 children (31 cases

and 31 controls) were enrolled. Children suffering from β thalassemia major aged >3 years on regular blood transfusions or with at least 20 transfusions whatever is later were included as cases. β thalassemia major was diagnosed based on following criteria in addition to necessity for repeated blood transfusions since their infancy: i) both parents' carriers of β thalassemia trait; ii) severe anemia during infancy along with characteristic appearance of peripheral blood film and unusually high levels of fetal hemoglobin.

Age and sex matched non-thalassemic children were included as controls. Thalassemia minor/traits were ruled out in controls by HbA_2 estimation. All controls were hospitalized for short-term illnesses.

Exclusion criteria

Patients having conditions causing hyperlipidemia like abnormal thyroid function tests, deranged fasting blood sugar and obesity were excluded from the study. Also, patients on prolonged fasting due to any illness were excluded. Basic demographic data from both study groups and disease related data in case of thalassemia group was collected. In addition, following observations were made and outcome assessor was blinded (Radiologist and Biochemists) to the thalassemic status of study participants.

Measurements of carotid intima-media thickness

To measure carotid intima-media thickness, high-resolution ultrasound study was done using high frequency linear probe (5-12 MHz) with Toshiba Nemio 30 (Toshiba Corp., Minato, Tokyo, Japan). Intima-media thickness of near and far wall for both common carotid arteries were measured in the distal 1-1.5 cm. Different scanning angles (anterolateral, posterolateral and mediolateral) were adopted.[13] Four measurements were taken for each common carotid artery (two for near wall and two for far wall) and average was recorded. All the measurements were recorded by same trained radiologist.

Biochemical parameters

A single 5-8 mL blood sample was collected from antecubital vein between 8 am to 10 am in sitting position after 12 h of fasting. Following biochemical investigations were carried out in all study participants: i) total cholesterol, triglycerides, high-density lipoproteins (HDL), LDL, oxidized LDL were measured by colorimetric method on fully automatic analyzer 902 HITACHI (Hitachi Ltd., Tokyo, Japan); ii) lipoprotein (a) (lipo a) was measure by ELISA on TECAN ANALYER (Tecan Trading AG, Männedorf, Switzerland); iii) serum ferritin was done by chemiluminiscence on ADVIA CENTAUR (CP) (Siemens, Munich, Germany). Prior 3-4 values of serum ferritin in addition were noted if available and average of all these were taken as the representative serum ferritin; iv) lipid peroxidation product MDA was done by the method forwarded by Ohkawa *et al*.[14] Principle: lipid peroxidation product reacts with thiobarbituric acid (TBA) to give pink chromophobe, which is measure at 535 nm; v) total antioxidant status was estimated by using total antioxidant kit. Principle: 2,2 Azinodi 3 ethyl benthizoline sulphonate (ABTS) is incubated with peroxide (methylglobulin) and H_2O_2 to produce the radical cation ABTS. This has a relatively stable blue-green color, which is measure at 600 nm. Antioxidants in sample cause suppression of color production to a degree, which is proportional to their concentration; vi) fasting blood sugar was measured by colorimetric method on fully automatic analyzer 902 HITACHI (Hitachi Ltd.); vii) thyroid profile was done by chemiluminiscence on ADVIA CENTAUR (CP) (Siemens).

Statistical analysis

Data of cases and controls was expressed as mean and standard deviations unless otherwise indicated and was compared between groups by unpaired Student's *t*-test or Mann-Whitney test depending upon normality of data. The dichotomous data was compared using chi-square test. Bivariate relations between outcome variables were evaluated using Pearson correlation coefficient. Multiple regression analysis was used to determine significant determinants of CIMT. Subgroup analysis according to age was also performed. Statistical significance was defined as P value <0.05. All statistical analysis was performed using SPSS version 17 (IBM Corp., Armonk, NY, USA).

Results

Basic demographic data of the cases and controls were matched (Table 1). Thalassemic patients age of diagnosis and treatment details thereof are depicted in Table 2. Primary outcome variable *i.e*., mean CIMT in cases was 0.69±0.11 mm and in controls, CIMT was 0.51±0.07 mm. 95% confidence interval for the mean difference between two groups were 0.058 to 0.153, with P value of 0.00 (P<0.001) which is highly significant with students independent t test (Table 3 and Figure 1).

Secondary outcome measures were compared between cases and controls using Student's independent t-test (Table 4). Oxidized LDL (EU/mL) was increased in cases

Table 1. Basic demographic data.

Sr. No.	Parameter	Mean±SD	CI	P value
1	Age at enrollment (y)		–2.21 -1.01	0.459
	Case	7.33±3.33		
	Control	7.93±3.02		
2	Height (cm)		–2.21-1.01	0.618
	Case	112.6±16.36		
	Control	114.4±10.92		
3	Weight (kg)		–3.75-2.98	0.908
	Case	20.41±7.11		
	Control	20.59±5.17		
4	BMI (m^2)		–0.328-1.67	0.184
	Case	16.31±2.23		
	Control	15.64±1.65		
5	Sex	Male	Female	0.437
	Case	17 (54.8%)	14 (45.2%)	
	Control	20 (64.5%)	11 (35.5%)	

SD, standard deviation; CI, confidence interval; BMI, body mass index.

Table 2. Treatment profile of β thalassemia major patients (cases).

Sr. No.	Parameter	Mean±SD	Range	Median
1	Age at diagnosis (y)	0.68±0.38	0.3-1.13	0.6
2	Age at first transfusion (y)	0.71±0.5	0.1-2	0.5
3	Duration since first transfusion (y)	6.8±3.44	2.25-14.00	5.25
4	Age at start of chelation (y)	2.81±2.52	0.5-9.1	2.03

SD, standard deviation.

as compared to controls. Mean oxidized LDL in cases was 39.3±34.4 (range 14.4 to 160) while in controls was 23.9±13.4 (range 12 to 70), and the difference between two groups is statistically significant (P=0.02, t-test). MDA, which is lipid oxidation product, was also increased in β thalassemic patients as compared to controls. Mean MDA (nmol/L) was 10.0±3.27 (4.41 to 17.48) while in controls 6.87±4.55 (1.5 to 17.9), which is statistically significantly higher in the cases than the control group (P=0.002, t-test). Mean total anti oxidant status (nmol/L) in cases was 1.63±0.39 (range 1.4 to 3.6) while in controls was 1.23±0.15 (range 1-1.5), however the difference is non significant (P=0.348). Mean lipo a in cases was 14.9±16.93 (range 1.2 to 52.3) while in controls was 16.98±14.62 (range 2.5 to 61.9) and the difference is statistically not significant. Among lipid profile (HDL, LDL, total cholesterol) difference between cases and controls was not statically significant. But among lipid profile, triglyceride (TG) levels (mg/dL) dif-

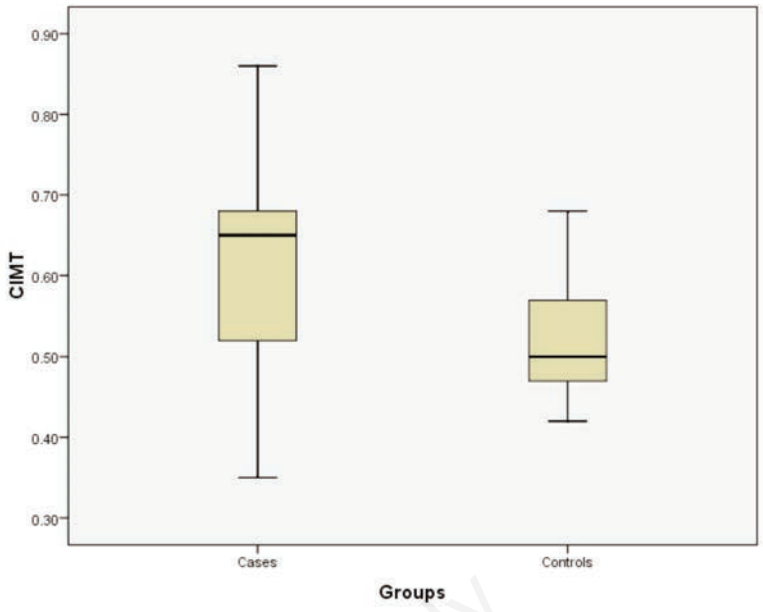

Figure 1. Mean carotid intima-media thickness (CIMT) in cases *vs* controls.

Table 3. Primary outcome measure in study population.

Sr. No	Parameter	Mean with SD	Range	Median	Mean diff.	Mean SE	CI	P value
			CIMT (mm)					
1	Case (n=31)	0.69±0.11	0.35-0.86	0.65	0.106	0.023	0.058-0.153	<0.001
	Control (n=31)	0.51±0.07	0.42-0.68	0.58				

SD, standard deviation; SE, standard error; CI, confidence interval; CIMT, carotid intima-media thickness.

Table 4. Secondary outcome measures in study population.

Sr. no	Parameter	Mean±SD	Range	Median	Mean diff.	SE of mean	CI	P value
1	Oxidized LDL (EU/mL)				15.41	6.64	2.12-28.7	0.026
	Case	39.32±34.4	14.4-160	20.4				
	Control	23.90±13.44	12-70	18.40				
2	MDA (nmol/L)				3.204	1.00	1.18-5.21	0.002
	Case	10.08±3.27	4.41-17.48	10.02				
	Control					6.87±4.55	1.50-17.9	4.5
3	Total anti oxidant status (mmol/L)				0.396	0.075	0.244-0.547	0.348
	Case	1.63±0.39	1.4-3.6	1.6				
	Control	1.23±0.15	1-1.5	1.21				
4	Lipoprotein (a) (mg/dL)				–2.04	3.93	–9.92-5.83	0.439
	Case	14.9±16.93	0-52.3	7.60				
	Control	16.98±14.62	2.5-61.9	11.30				
5	HDL (mg/dL)				1.48	3.33	–5.19-8.16	0.658
	Case	30.23±9.95	13-59	31.0				
	Control	28.7±15.69	8-81	24				
6	LDL (mg/dL)				–4.58	5.75	–16.0-6.92	0.430
	Case	56.7±15.1	31-92	55				
	Control	61.3±28.2	18-132	55				
7	Total cholesterol (mg/dL)				–5.74	8.07	–21.89-10.41	0.481
	Case	115.5±20.5	77-155	117				
	Control	121.2±40.0	55-214	110				
8	TG (mg/dL)				–50.5	22.20	–94.93–-6.101	0.028
	Case	126.5±53.07	39-249	122				
	Control	177.1±111.6	43-445	147				

SD, standard deviation; SE, standard error; CI, confidence interval; LDL, low-density lipoprotein; MDA, malondialdehyde; HDL, high-density lipoproteins; TG, triglycerides.

fered in both the groups significantly (0=0.02), mean TG in cases being lower 126.5±53 (range 39 to 249) than the controls 177±111 (range 43 to 445). In secondary outcome measures, all parameters show normal distribution except for oxidized LDL, MDA, total antioxidant status and lipo a. For these data, Mann-Whitney test was applied which showed similar results for oxidized LDL (P=0.02), MDA (P=0.001), and Lipoprotein (a) (P=0.145). However, total antioxidant status (P<0.001) showed significant difference statistically between two groups.

Iron status of thalassemia major patients was evaluated. Mean serum ferritin was 4115±2247 (ng/mL) (range 205 to 9773) and mean serum iron in cases was 196±58 (mol/dL) (range 25 to 325).

CIMT in thalassemia major patient group was correlated with all secondary outcome measures (Table 5). For normally distributed data, Pearson's correlation test was applied and for skewed data (lipo a, oxidized LDL, serum ferritin, total antioxidant status, MDA), Spearman's test was applied. CIMT correlated positively with on lipo a with correlation coefficient of 0.367 and P value of 0.04, which is statistically significant (Figure 2).

Regression analysis of CIMT with all secondary outcome measures was done (Figure 3). Single most predictor of CIMT of all secondary outcome measures was total antioxidant level with -value 0.394, which is significant (P=0.028).

Discussion

In our study, CIMT was significantly increased in β thalassemia major patients' as compared to healthy controls.

Cheung *et al.*[15] (mean age of cases 21±5.6 years) and Tantawy *et al.*[16] (mean age of cases 18.4±6.18 years) both in their study showed that the CIMT of thalassemic patients was significantly increased compared to controls. Even though our study population is younger with a mean age of cases being 7.33±3.33 years and controls 7.93±3.02 years, we found similar results of significantly increased CIMT in β thalassemic patients than in controls (P<0.001).

In studies on pediatric population, Limsuwan *et al.*[17] analyzed CIMT in β thalassemia children (mean age 10.1±2.7 years) who were treated conventionally or with bone marrow transplantation. They found CIMT to be increased in conventionally treated β thalassemia major patients and suggested early atherosclerotic changes in these patients. Dogan *et al.*[18] also studied CIMT in 33 thalassemic patients (22 boys, 11 girls with median age of 8 y). They found median CIMT in thalassemic patients to be significantly higher *i.e.*, 0.87 mm in cases than in controls 0.74 mm (P<0.05). They also suggested CIMT as an early non-invasive marker of cardiovascular morbidity and mortality.

Our study in concurrence with the above quoted studies thus suggests than CIMT is increased in β thalassemic disease and is an early noninvasive marker for assessment of atherosclerosis in the diseased population.

Secondary outcome measures

Oxidized low-density lipoproteins

Oxidized LDL is oxidative modification product of circulating LDL that could represent an event leading to atherogenesis.[16] We found significantly increased level of oxidized LDL in β thalassemia patients as compared to controls. Brizzi *et al.*[19] investigated the levels of oxidized LDL antibodies (OLAB) in 75 β thalassemia patients with a median age of 21 y (range 2.5-

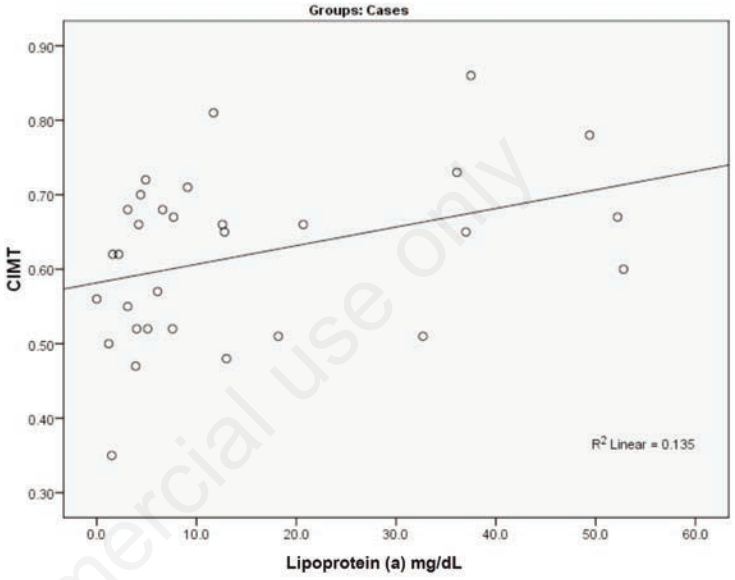

Figure 2. Correlation of carotid intima-media thickness (CIMT) with lipoprotein (a).

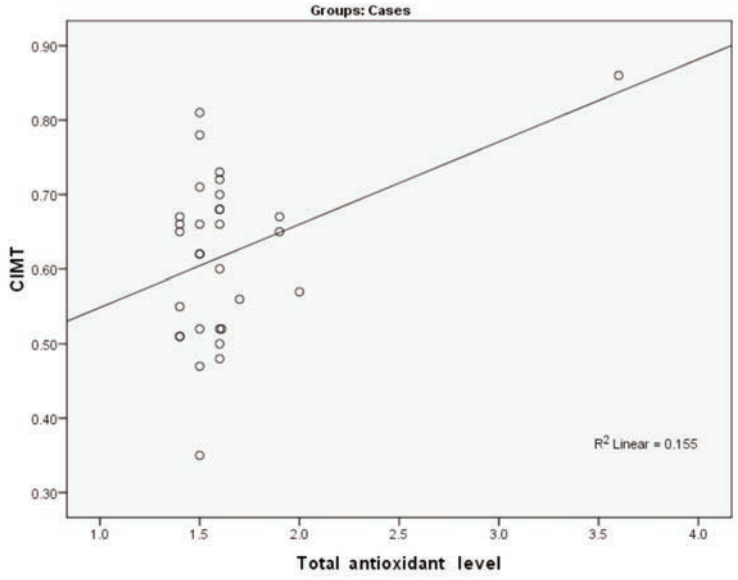

Figure 3. Regression analysis depicting correlation between carotid intima-media thickness (CIMT) and total antioxidant level.

42 y) and compared with 75 healthy controls with median age 24 y (range 18-34 y). However, considering β thalassemia patients to be having lower LDL-cholesterol than control, they also measured OLAB/cholesterol-LDL ratio in the study population, which indicates the real antibody response to oxidized LDL. OLAB/ cholesterol-LDL ratio was higher in thalassemic patients (6.8±3.4) than healthy controls (3.74±1.51) in their study.

Malondialdehyde

MDA is a marker of lipid peroxidation injury and plays an important role in premature atherosclerosis.[20] In our study, we found MDA levels (nmol/mL) to be significantly increased in β thalassemia patients as compared to controls. Giardini *et al*.[21] demonstrated that red blood cell MDA was significantly higher in β thalassemia patients as compared to controls. Livrea[8] showed that the mean concentrations of lipoperoxides evaluated as malondialdehyde/thiobarbituric acid (MDA/TBA) adducts, increased about twofold in their thalassemic patients with respect to controls. Livrea *et al*.[20] also found MDA to be increasd by two fold in β thalassemia intermedia patients as compared to controls.

Table 5. Correlation of carotid intima-media thickness with secondary outcome variables in cases.

	CIMT
CIMT	
Pearson's correlation	1
Sig. (2-tailed)	
N	31
Serum ferritin	
Correlation coefficient	0.017
Sig. (2-tailed)	0.930
N	31
Oxidized LDL	
Correlation coefficient	–0.210
Sig. (2-tailed)	0.258
N	31
MDA	
Correlation coefficient	–0.094
Sig. (2-tailed)	0.614
N	31
Total antioxidant level	
Correlation coefficient	0.166
Sig. (2-tailed)	0.373
N	31
HDL	
Pearson's correlation	–0.022
Sig. (2-tailed)	0.906
N	31
LDL mg dL	
Pearson's correlation	0.138
Sig. (2-tailed)	0.459
N	31
Total cholesterol	
Pearson's correlation	0.004
Sig. (2-tailed)	0.983
N	31
TG	
Pearson's correlation	–0.016
Sig. (2-tailed)	0.932
N	31
Lipoprotein (a)	
Correlation coefficient	0.367
Sig. (2-tailed)	0.042
N	31
Serum iron	
Pearson's correlation	0.048
Sig. (2-tailed)	0.799
N	31

CIMT, carotid intima-media thickness; Sig., significance; LDL, low-density lipoprotein; MDA, malondialdehyde; HDL, high-density lipoproteins; TG, triglycerides.

Total antioxidant status

In the present study, total antioxidant status was significantly greater in β thalassemia patients compared to controls. This however showed no significant difference in cases and controls with study population stratified into two age groups =8 or >8 years (P=0.16, P=0.64 in age groups =8 years and >8 years, respectively). Livera *et al*.[21] showed total serum antioxidant potential, measured as trolox equivalent antioxidant capacity (mmol/L) to be significantly decreased by 14% in patients with β thalassemia major. In another study by Livrea,[8] they showed 52% decrease in antioxidant level in patients with β thalassemia *versus* healthy controls.

Lipoprotein (a)

Lipoprotein (a) is an LDL like particle,[16] it is potentially thrombogenic and has been associated with increased cardiovascular risk. In our study lipo a value was higher in controls than our β thalassemia patients. However, the difference was statistically non-significant (P=0.145). Maioli *et al*.[22] showed low lipo a levels (apo A-I and apo B) in patients with homozygous β thalassemia. Median lipo a (apo a) levels in β thalassemia patients 15.3 mg/dL (1.4-55.7), in β thalassemia trait 11.9 mg/dL (1.7-179.2) and in controls was 18.9 mg/dL (0.7-156.8), which was not significant. Tantawy *et al*. showed lipoprotein levels to be relatively higher in β thalassemia patients as compared to controls, but this rise was not statistically significant (13.32 and 8.74 mg/dL, respectively, P=0.50). To best of our knowledge these are only two studies investigating lipo a levels in patients with β thalassemia major patients and our study results of decreased lipo a in β thalassemia disease are consistent with results of study by Maioli *et al*.[22] An impaired liver function might be a likely explanation for the decreased lipoprotein levels.

Lipid profile

Alerted lipid profile is thought to be a contributing factor for atherosclerosis in patients with β thalassemia major. Serum TG levels (mg/dL) were decreased in β thalassemia patients as compared to controls. Mean TG in cases was 126.5±53 (range 39 to 249), while in

controls mean was 177±111 (range 43 to 445) and the difference was statistically significant (0=0.02). Rest parameters of liplid profile are not statistically significant. Chrysohoou *et al*.[9] in their study found that majority of the thalassemic patients had blood lipid levels within the normal range with the exception of HDL cholesterol which was decreased in β thalassemia than the standard value.

Iron overload

Iron status was evaluated with serum ferritin and serum iron in study population. In our study, primary outcomre measure (CIMT) was not significantly correlated with serum ferritin (ρ=0.01, P=0.93). However, Tantawy *et al*.[16] and Dogan *et al*.[18] in their study on β thalassemia patients showed that mean CIMT value was positively correlated with serum ferritin (P=0.01). In our study CIMT value was not correlated significantly with serum ferritin. As compared to the above studies, our study population included younger patients. Hence, duration of disease might have influenced the results. Livrea *et al*.[20] showed that serum ferritin levels were positively correlated with the amount of MDA (ρ=0.41; P=0.007) and also showed a positive trend with conjugated diene (ρ=0.31; P=0.07) and protein carbonyls (ρ=0.35; P=0.054). In our study also, serum ferritin level was positively correlated with lipid peroxidation marker, oxidized LDL (P=0.006). However, serum ferritin showed no significant correlation with MDA (P=0.11).

Correlation of carotid intima-media thickness with secondary outcome variables

In the present study, when CIMT was correlated with all secondary outcome measures, lipo a (ρ=0.367, P=0.04) is the only outcome measure that was positively correlated with CIMT in β thalassemia major patients while none of the other secondary outcome measure was positively correlated with CIMT in control group and in regression analysis total antioxidant status showed predictability towards CIMT (R=0.39 and P=0.02). Brizzi *et al*.[19] found significant positive correlation between oxidized LDL/LDL-cholesterol ratio and triglycerides in β thalassemia patients (P<0.001). In our study we also found significant positive correlation between oxidized LDL and triglycerides in β thalassemia (P<0.007). In addition to TG, we also found significant positive correlation between oxidized LDL and total cholesterol (P<0.001) and lipo a (P<0.005). To the best of our knowledge, ours is only study looking for correlation of CIMT with oxidative stress markers.

Conclusions

Strengths and limitations of the study

This is the first study of its kind in literature showing correlation of CIMT with oxidative stress markers. We were able to include only 31 children in each study group. Also, there is wide range of age distribution in study groups [cases (3-14 years), controls (3.5-13.5 years)]. Overall, the study population was mainly young children and adolescents. Thus the duration of exposure to the disease and iron overload was limited which might have influenced the study results. Henceforth, the results of our study cannot be extrapolated to the general population at large. Our study results show CIMT as an early marker of atherogenesis in β thalassemia major. Timely screening of β thalassemia major patients by assessing a non invasive marker (CIMT) may have implications in prevention of cardiovascular disease in β thalassemia major patients. In the present study, oxidative stress markers are also increased in β thalassemia major patients and lipo a shows a positive correlation with CIMT. This points towards atherogenetic mechanisms in β thalassemia major and hence opens a wide arena of research where further studies are required to explore various pathogenetic mechanisms underlying atherosclerosis in β thalassemia major.

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